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The 3′-terminal region of the mRNAs for VSG and procyclin can confer stage specificity to gene expression in *Trypanosoma brucei*

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The variant surface glycoprotein (VSG) and procyclin are the respective major surface antigens of the bloodstream and the procyclic forms of *Trypanosoma brucei*. These proteins and their mRNAs are both the most abundant and absolutely characteristic of their respective life cycle stages. We show that the 3′-terminal region of these mRNAs regulates expression of a reporter gene in an inverse manner, depending on the developmental form of the parasite. In the case of VSG mRNA, the 97 nt sequence upstream from the polyadenylation site is responsible for these effects. The regulation occurs through a variation of mRNA abundance which is not due to a change in primary transcription. In the bloodstream form this effect is manifested by an increase in RNA stability, whereas in the procyclic form it seems to be related to a reduction in the efficiency of mRNA maturation. The 3′-end of VSG mRNA can obviate the 5- to 10-fold stimulation of transcription driven by the procyclin promoter during differentiation from the bloodstream to the procyclic form. The predominance of post-transcriptional over transcriptional controls is probably linked to the organization of the trypanosome genome in polycistronic transcription units.

Key words: differentiation/gene expression/post-transcriptional controls/RNA stability/*Trypanosoma brucei*

Introduction

During their life cycle, parasites such as *Trypanosoma brucei* undergo rapid and important morphological and physiological transformations to adapt to their changing environment. In particular, during differentiation of the bloodstream form to that characteristic of the midgut of the tsetse fly, termed the procyclic form, the trypanosomes quickly replace their major surface antigen, VSG (variant surface glycoprotein), by another predominant surface glycoprotein, called procyclin or PARP (procyclic acidic repetitive protein) (Roditi *et al.*, 1989; Ziegelbauer *et al.*, 1990). Although their amino acid sequences and their structures differ, these proteins seem to be functionally analogous and probably protect the parasite against host lytic elements (Roditi and Pearson, 1990). Analysis of the replacement of VSG by procyclin represents a useful model to study the genetic mechanisms of differentiation. Indeed, these proteins and their mRNAs are both the most abundant and absolutely characteristic of their respective developmental forms. In addition, differentiation from the bloodstream to the procyclic form can be mimicked *in vitro* under controlled conditions. For example, when quiescent forms of the bloodstream stage, called stumpy forms, are incubated at 27°C in the presence of citrate/*cis*-aconitate, differentiation occurs efficiently and synchronously (Ziegelbauer *et al.*, 1990). Under these conditions disappearance of VSG mRNA and simultaneous accumulation of procyclin mRNA occur very rapidly, before the first cell division (Pays *et al.*, 1993).

As is probably the case for the majority of genes in trypanosomes (reviewed in Clayton, 1992; Pays, 1993; Pays *et al.*, 1994), the VSG and procyclin genes are contained in polycistronic transcription units. The VSG unit, generally termed the VSG expression site, is a 45–60 kb telomeric sequence which includes a battery of expression site-associated genes (ESAGs) located in front of the VSG gene (Cully *et al.*, 1985; Kooter *et al.*, 1987; Alexandre *et al.*, 1988; Pays *et al.*, 1989b; Lips *et al.*, 1993). Although several potentially functional VSG units are present in the genome, only a single one is active at a time (Zomerdijk *et al.*, 1990; Jefferies *et al.*, 1991). There are two distinct and diploid procyclin transcription units, each with a size of ~10 kb and each containing a pair of procyclin genes located upstream from a couple of procyclin-associated genes (PAGs) (Clayton *et al.*, 1990; Rudenko *et al.*, 1990; Berberof *et al.*, 1991; Koenig-Martin *et al.*, 1992). These units are not telomeric and are probably all active simultaneously (Koenig *et al.*, 1989; Clayton *et al.*, 1990). Presumably to achieve a high level of expression, the VSG and procyclin units are transcribed by a RNA polymerase sharing characteristics with that transcribing ribosomal genes (reviewed in Chung *et al.*, 1992).

Little is known about the controls that ensure mutually exclusive activation of the VSG and procyclin units. Their transcription promoters have been characterized (Clayton *et al.*, 1990; Pays *et al.*, 1990; Zomerdijk *et al.*, 1990; Gottesdiener *et al.*, 1991; Rudenko *et al.*, 1990) and are at least partially active during both stages of the parasite life cycle, even though there is a rigorous stage-specific control of the expression of their respective units (Pays *et al.*, 1989a, 1990; Zomerdijk *et al.*, 1990; Jefferies *et al.*, 1991; Rukenko *et al.*, 1994). Constitutive transcription appears to be attenuated by regulation of RNA elongation (Alexandre *et al.*, 1988; Pays *et al.*, 1990; Vanhamme *et al.*, 1995). This form of transcriptional control has only

been observed in the case of the VSG and procyclin units. In all other cases the polycistronic units appear to be constitutively transcribed.

Despite an apparent absence of control of primary transcription, most trypanosome genes analysed to date show stage-specific expression. Typically, contiguous genes belonging to the same unit are often differentially regulated during development of the parasite (examples in Gibson *et al.*, 1988; Revelard *et al.*, 1993; reviewed in Clayton, 1992; Pays, 1993). In addition, at a given developmental stage the levels of mRNAs from genes of the same unit can exhibit large differences: this is particularly evident within the VSG unit in the bloodstream stage, where a 700-fold difference has been measured between VSG and ESAG 1 mRNA levels (Cully *et al.*, 1985). Therefore, it is clear that post-transcriptional controls are very important in regulation of individual gene expression. This conclusion seems also to apply to the VSG and procyclin units, since transcriptional controls do not account completely for the typical stage-specific expression of these units.

In trypanosomes post-transcriptional processes involve coupled events of *trans*-splicing and polyadenylation (LeBowitz *et al.*, 1993; Hug *et al.*, 1994; Matthews *et al.*, 1994; Schürch *et al.*, 1994; Vassella *et al.*, 1994). These events appear to depend on the nature of intergenic sequences, particularly the presence of polypyrimidine tracts (Huang and Van der Ploeg, 1991; Hug *et al.*, 1994; Matthews *et al.*, 1994; Schürch *et al.*, 1994; Vassella *et al.*, 1994). Therefore, it is not surprising that untranslated regions (UTRs) downstream and upstream of the gene open reading frames may influence the level of expression of genes when assayed in reporter constructs (Jefferies *et al.*, 1991; Hug *et al.*, 1993; Aly *et al.*, 1994; Hehl *et al.*, 1994). In particular, the 3′-end region of VSG mRNA allowed efficient expression of a reporter gene in the bloodstream form (Jefferies *et al.*, 1991). This region is composed of both a polypyrimidine stretch and conserved sequence elements. The aim of this study was to define the sequences of the 3′-end region that influence gene expression and to examine the mechanism of this regulation. Our results indicate that a 97 nt region upstream of the polyadenylation site is both necessary and sufficient to confer stage specificity to gene expression. The up-regulation in the bloodstream form is at least partially achieved by a modulation of mRNA stability, while another control, presumably at the level of RNA maturation, appears to account for the down-regulation that occurs during differentiation into the procyclic form. Significantly, an inverse pattern of regulation was obtained with the 3′-end region of procyclin mRNA. In the transcription units for the major stage-specific antigens, such post-transcriptional controls probably add to transcriptional regulation to ensure a strict stage specificity of gene expression.

Results

The 3′-terminal region of VSG mRNA determines a stage-specific level of gene expression

The transcription promoter of the AnTat 1.3A VSG expression site was inserted in front of the bacterial gene for chloramphenicol acetyl transferase (CAT) in a plasmid construct termed pD5 (Jefferies *et al.*, 1991). CAT activity was readily detectable when this construct was transfected into the procyclic form, but in the bloodstream form no activity was observed unless a 340 bp fragment from the 3′-end of AnTat 1.10 VSG cDNA was placed downstream of the CAT gene (Jefferies *et al.*, 1991). This result suggested that the 3′-UTR of VSG mRNA is able to up-regulate gene expression in the bloodstream form. As shown in Figure 1A (lines 1 and 2), in transient CAT activity assays the presence of this sequence reduced gene expression in the procyclic form, while increasing it in the bloodstream form. Overall, a 19-fold difference was observed between the two forms. In order to determine if this was true for the 3′-ends of different VSG mRNAs, as well as to determine precisely the region responsible for the effect, different fragments from the 3′-end and environment of the telomeric IsTat 1.1 VSG gene of *T.brucei* (Aline and Stuart, 1989) were placed downstream of the CAT gene in the pD5 construct. As shown in Figure 1A (lines 3 and 4), a 1.25 kb *Hae*III–*Eco*RI fragment covering the last 97 bp of the VSG mRNA region together with the 3′ environment of the gene was able both to stimulate CAT activity in the bloodstream form and inhibit this activity in the procyclic form, each effect being ~3-fold. The first 138 bp of the sequence (*Hae*III–*Hin*dII fragment) were found to be essentially responsible for these effects, while the 3′ gene environment (1.1 kb *Xba*I–*Eco*RI fragment) was ineffective (Figure 1A, lines 5 and 6 and 7 and 8 respectively). The combination of these data indicates that modulation of gene expression is due to sequence elements contained within the last 97 nt before the polyadenylation site of VSG mRNA. This region contains both a polypyrimidine tract and some elements (8mer and 14mer) that are conserved in all VSG mRNAs analysed to date (at least 45 different sequences) (Figure 1B). In the following experiments described in this study, the 138 bp *Hae*III–*Hin*dII fragment from the IsTat 1.1 gene was used as the 3′-end region.

Opposite effects are conferred by the 3′-end region of procyclin mRNA

If physiologically significant, the effects obtained with the 3′-end region of the VSG mRNA should be different when using sequences from genes that are differently regulated *in vivo*. Therefore, in the same system we analysed the effects of the 3′-end region of procyclin mRNA (inverse regulation *in vivo*) and of β-tubulin mRNA (minor regulation *in vivo*). A 480 bp fragment covering the last 223 nt of α-procyclin mRNA and the 3′ flanking genomic sequence was inserted downstream of the CAT gene in pD5. This fragment was found to regulate gene expression in an opposite fashion to that of the VSG gene fragment. As shown in Figure 1A (lines 9 and 10), a >8-fold difference in favour of the procyclic form was observed in this case. In contrast, a much lower effect was obtained with a 300 bp fragment covering the last 150 nt of β-tubulin mRNA and the 3′ flanking genomic sequence. The presence of this fragment resulted in only a 1.4-fold increase in gene expression in the bloodstream form (Figure 1A, lines 11 and 12). In conclusion, the results presented in Figure 1A are in accordance with those expected from the patterns of gene expression

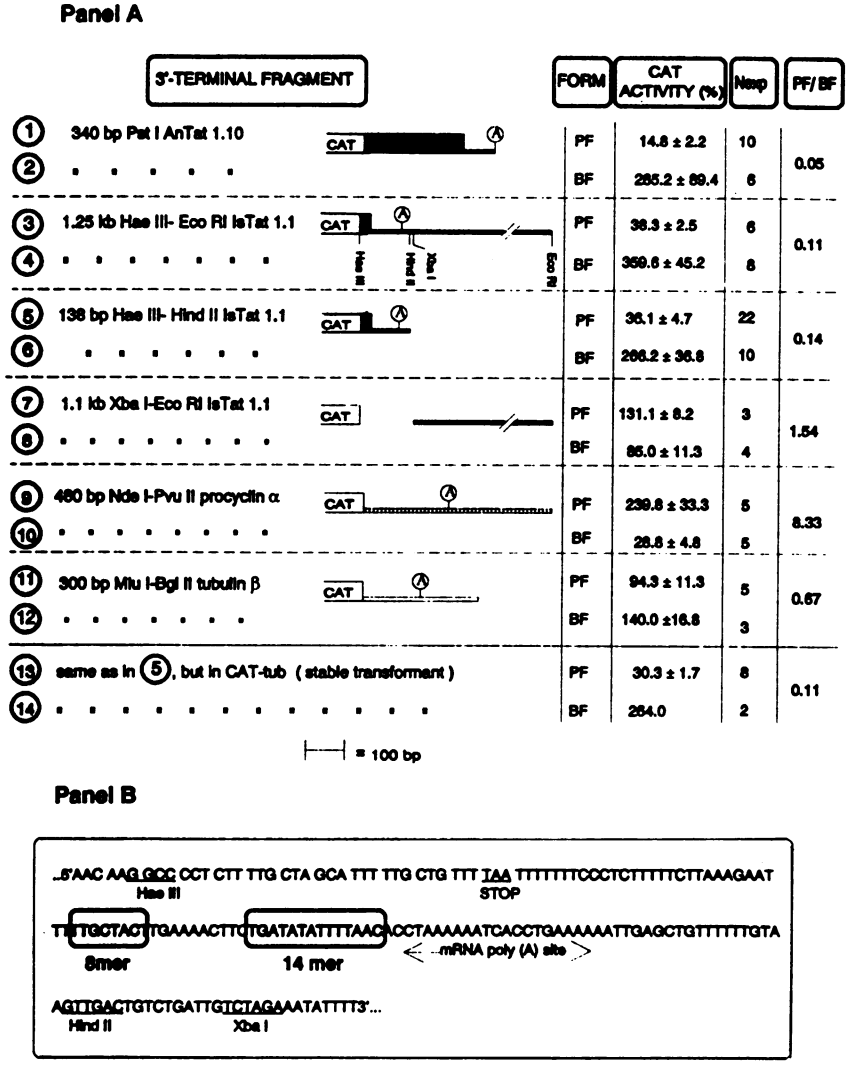

Fig. 1. (**A**) Effect of fragments from the 3′-end region of different genes on CAT activity assays in *T.brucei*. The activity was expressed as a percentage of that measured in the absence of trypanosome 3′-end region. All measurements were performed in transient assays, except in lines 13 and 14, where permanent CAT activity was monitored. The black box and thick line to the right of the CAT gene refer respectively to the end of the VSG open reading frame and the downstream non-coding sequence. BF and PF, bloodstream and procyclic forms; Nexp, number of experiments; A, polyadenylation site. The PF/BF values represent the ratios between the percentages of CAT activity (with UTR versus without UTR) in PF and BF. (**B**) Nucleotide sequence of the 3′-end region of the IsTat 1.1 VSG gene (Aline and Stuart, 1989).

in vivo, suggesting that the regulation observed with the 3′-end region of mRNAs is physiologically relevant.

Delimitation of the sequence responsible for control of gene expression

As shown in Figure 2 (lines 1 and 2), inversion of the 3′-end region of VSG mRNA abolished regulation of gene expression in both bloodstream and procyclic forms. Therefore, this effect is strand dependent. Different deletions and substitutions were then performed to delimit the regulatory element. We concentrated first on the conserved 14mer and 8mer. The deletion of either of these blocks led to a loss of regulation (Figure 2, lines 3–6). Curiously, simultaneous deletion of both blocks appeared to conserve some detrimental activity in the procyclic form (Figure 2, lines 7 and 8). Inversion of the 14mer in its environment or its substitution by a random sequence abolished regulation (Figure 2, lines 9–12). However, these results did not imply that the 14mer alone conferred regulation of gene expression, because if inserted just before the polyadenylation site of β-tubulin mRNA, the 14mer was unable to reproduce the effects of the VSG 3′-end sequence (Figure 2, lines 13 and 14). The spacing between the 8mer and 14mer appeared to be more important than the nucleotide sequence between them. As shown in Figure 2 (lines 15–18), some of the regulation (3-fold difference between stages) was conserved when a random sequence replaced that present between the 8mer and the 14mer, while the difference was only 1.3-fold if the region between the elements was deleted. Finally, the totality of the 3′-end region, including the last 10 codons of the open reading frame, appeared to be required for activity. Indeed, oligonucleotides covering either the 8mer–14mer region or the complete 3′-UTR did not influence gene expression (lines 19–22). In conclusion, the entire 97 nt region upstream of the polyadenylation site, with possibly no stringent sequence requirement in the region between the 8mer and the 14mer, was necessary to achieve the opposite stage-

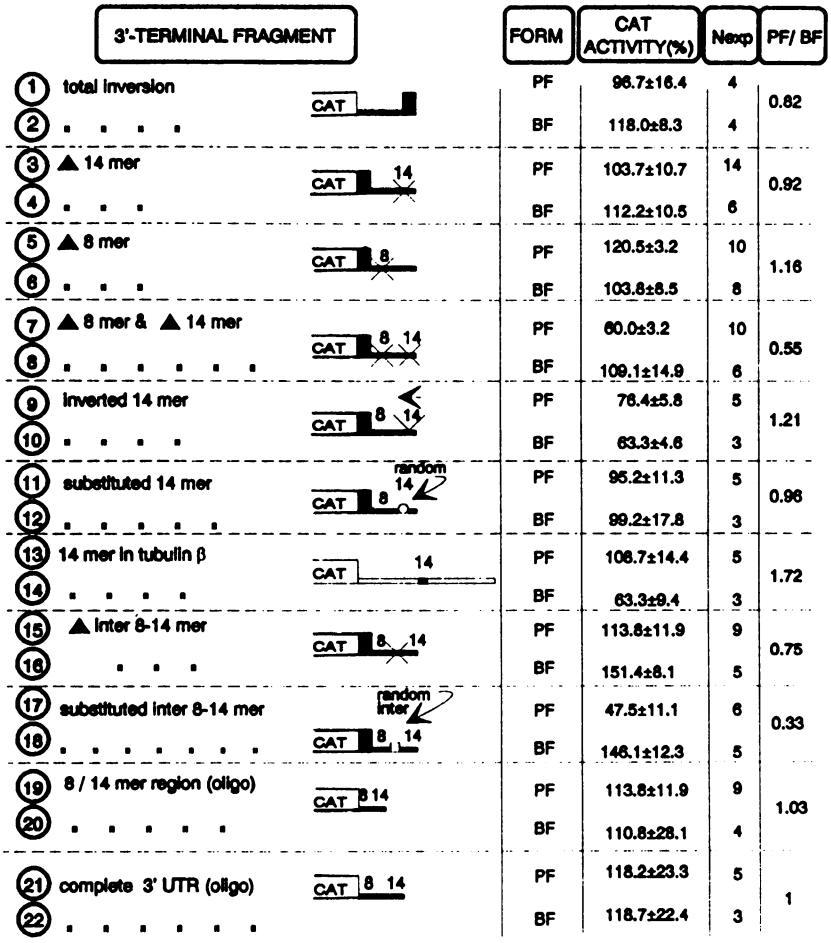

Fig. 2. Dissection of the sequence required for regulation. Abbreviations as in Figure 1A.

specific regulation. This region includes not only the 3′-UTR, but also the last 10 codons of the VSG gene, which are relatively well conserved between VSG genes.

Regulation of gene expression is post-transcriptional

In order to determine at which level regulation of CAT activity is achieved, stable trypanosome transformants were created through targeting of a plasmid reporter construct to the tubulin locus of the *T.brucei* procyclic form. Figure 3 shows the structure of this construct, termed CAT-tub. It contains the CAT gene preceded by the procyclin gene promoter and splice acceptor sites followed by the gene for resistance to hygromycin (HYG^R) and flanked by the procyclin splice site and polyadenylation region of the β-tubulin gene. The CAT gene was either devoid of trypanosome 3′-UTR (C, for control) or provided with the 3′-end region of VSG mRNA (V, for VSG). In both cases a single *Bam*HI site was present in the construct, between the CAT and HYG^R genes. After linearization by cleavage at the *Mlu*I site present in the 3′-UTR of the β-tubulin gene, these constructs were integrated into the tubulin gene array by homologous recombination (Lee and Van der Ploeg, 1990; ten Asbroek *et al.*, 1990; Eid and Sollner-Webb, 1991). Transformants with the same number of integrated copies were selected to allow a reliable comparison of the results. The data presented in Figure 3 show that in both selected transformants a tandem integration occurred. Hybridization with the CAT gene probe revealed the expected 5′-flanking *Bam*HI fragment of 5.7 or 5.9 kb (depending on the construct) (arrow), in addition to the tandem-specific 6.9 or 7.1 kb *Bam*HI fragment (asterisk). Similarly, hybridization with the HYG^R probe revealed the expected 3′-flanking *Bam*HI fragment of 5 kb (arrow), in addition to the tandem-specific *Bam*HI fragment (asterisk). As expected, after *Mlu*I digestion the two copies of the construct were superimposed, thus doubling the intensity of labelling (Figure 3, lanes M). Hybridization with tubulin-specific probes confirmed the integration (data not shown). These procyclic transformants were submitted to cyclical transmission through tsetse flies, to obtain the corresponding bloodstream forms (Jefferies *et al.*, 1993). The hybridization patterns in these forms were indistinguishable from those presented in Figure 3, indicating that no DNA rearrangement occurred during cyclical transmission (data not shown).

The levels of permanent CAT activity were measured in these transformants. As shown in Figure 1 (lines 13 and 14), the stage-specific differences conferred by the 3′-end region were similar to those observed in the transient activity assays.

The nuclei from both forms of each transformant were incubated *in vitro* to estimate the effect of the presence of the 3′-end region on the rate of primary transcription of the CAT gene at each developmental stage. As shown

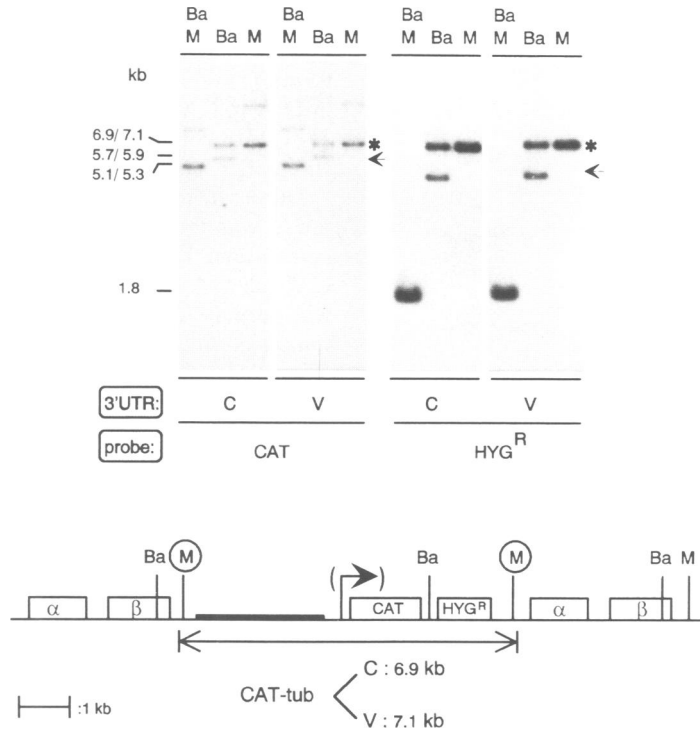

Fig. 3. Integration of the CAT constructs into the tubulin locus. Southern blots of genomic DNA digests of the CAT-tub C and V transformants were hybridized with the CAT and HYG^R probes. The fragments indicated by an asterisk and an arrow respectively span a tandem insertion of the construct and the flanking environment. In the map, the extent of the CAT-tub construct, flanked by the encircled *Mlu*I site used for plasmid linearization, is shown in its genomic environment. The thick line represents plasmid DNA. The arrow between parentheses refers to the procyclin promoter, activated in the procyclic form. α and β refer to the tandem tubulin genes. Ba, *Bam*HI; M, *Mlu*I.

in Figure 4, no difference was observed at this level (compare C and V lanes; quantitative measurements of the data are presented below the lanes). Therefore, the difference in CAT activity conferred by the 3′-end region must be ascribed to regulation occurring downstream of the primary transcription event.

Interestingly, these experiments showed that in the bloodstream form the procyclin promoter contained in the construct is bypassed by the α-amanitin-sensitive RNA polymerase at work in the tubulin locus (compare lanes – and + α-amanitin in BF). In contrast, the procyclin promoter was activated in the procyclic form, as evidenced both by the 5-fold increase in transcription of the construct and by its resistance to α-amanitin (compare PF versus BF in each panel).

It is remarkable that at each developmental stage the presence of the 3′-end region achieved the same modulation of CAT activity when assayed in different systems (transient and stable assays) where the CAT gene was transcribed by different RNA polymerases (α-amanitin-sensitive or -resistant) recruited by different promoters (VSG, procyclin or tubulin). These observations further confirmed that the regulatory effects are independent of the primary transcription process.

Modulation of CAT activity is due to a differential accumulation of CAT mRNA

mRNAs of the CAT and HYG^R genes from both CAT-tub C and V transformants were analysed by cDNA sequencing (data not shown; see Berberof *et al.*, 1995). As expected, these transcripts were *trans*-spliced at the procyclin splice acceptor site present in front of both genes, while poly-

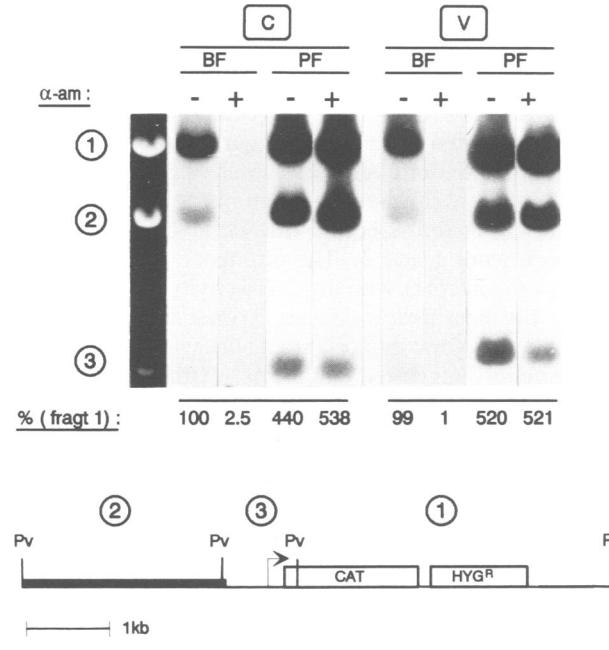

Fig. 4. The presence of the 3′-end region does not affect primary transcription of the CAT construct. Run-on transcription assays were conducted in bloodstream (BF) and procyclic forms (PF) of the CAT-tub C and V transformants, in the presence or absence of 1 mg/ml α-amanitin (α-am) as indicated. The numbers refer to the position of the *Pvu*II fragments in the map below. Hybridization to fragment 1, which spans both the CAT and HYG^R genes, was quantitated by liquid scintillation counting and the measurements are listed below the blots (100% = 1806 c.p.m.). Pv, *Pvu*II.

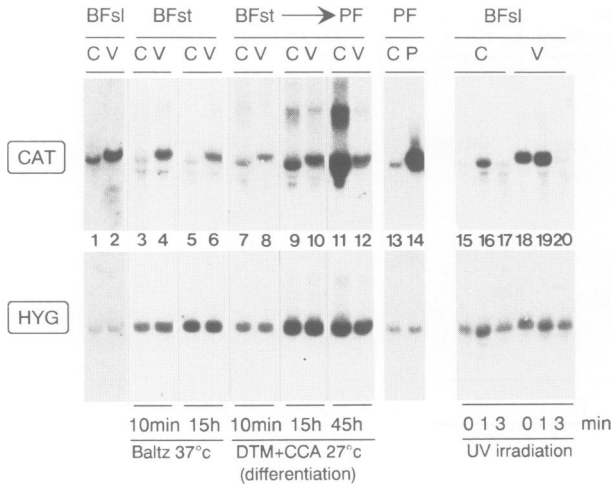

Fig. 5. Steady-state levels of CAT and HYG^R mRNAs in bloodstream and procyclic forms of the CAT-tub transformants. Total RNA (10 μg) from either bloodstream (BF: sl, slender; st, stumpy) or procyclic forms (PF) of the CAT-tub C and V transformants was hybridized with the CAT and HYG^R probes. The experimental treatments are described in the text.

adenylation occurred either in the VSG polyadenylation region (V transformants) or in a plasmid DNA site located 111 bp upstream of the HYG^R splice site (C transformants). Interestingly, in the V transformants polyadenylation occurred in the same region, irrespective of the deletion of either the 14mer or 8mer (data not shown). Due to the different locations of the polyadenylation site, the sizes of the different CAT cDNAs analysed were respectively ~1700 nt for V and 1580 nt for C, from the spliced leader to the first residue of the poly(A) tail. Accordingly, the size of the CAT mRNA appeared to be ~100 nt higher in CAT-tub V than in CAT-tub C, while the HYG^R mRNA was identical in both transformants (Figure 5). In bloodstream forms the amount of CAT mRNA was reproducibly found to be 2.5- to 4-fold more abundant in CAT-tub V than in CAT-tub C, while the HYG^R mRNA levels were similar (Figure 5, lanes 1 and 2). The difference in CAT mRNA levels was in keeping with the CAT activity measurements, both in transient assays (Figure 1, lines 5 and 6) and in the stable transformants (Figure 1, lines 13 and 14). This result strongly suggested that the up-regulation exerted by the 3′-region of VSG mRNA in the bloodstream forms is due to an increase in steady-state levels of mRNA.

In order to monitor the regulation conferred by the 3′-region of VSG mRNA during differentiation into the procyclic form, the bloodstream cell population was enriched in differentiation-competent stumpy forms by prolonged growth in immunosuppressed mice (Ziegelbauer *et al.*, 1990; Pays *et al.*, 1993). When incubated at 37°C in Baltz medium, which allows cultivation of bloodstream forms (Baltz *et al.*, 1985), these cells showed the same CAT mRNA difference as found in proliferative bloodstream forms (slender forms) (Figure 5, lanes 3–6 for two incubation periods, 10 min and 15 h). In contrast, when incubated under conditions for differentiation into the procyclic form (27°C in DTM medium supplemented with citrate/*cis*-aconitate; Ziegelbauer *et al.*, 1990), this difference was immediately abolished and was progressively inverted (Figure 5, lanes 7–12 for three incubation periods, 10 min, 15 h and 45 h). After 45 h of this treatment the cells which began to proliferate as procyclic forms (Pays *et al.*, 1993) contained ~4-fold more CAT mRNA in CAT-tub C than in CAT-tub V (Figure 5, lanes 11–12). In contrast, the levels of HYG^R mRNA remained similar in C and V during the entire period of the treatment.

During differentiation the level of CAT mRNA in CAT-tub V was found to be only slightly increased (compare lanes 4 and 12), contrasting with the 15-fold increase in CAT-tub C (compare lanes 3 and 11). The latter increase was partially due to a 4- to 5-fold stimulation of primary transcription linked to activation of the procyclin promoter (Figure 4), as also revealed by the increase in HYG^R mRNA levels (Figure 5, lanes 7–12). Taken together, these observations suggested that the presence of the 3′-region of VSG mRNA is able to counteract transcriptional stimulation due to activation of the procyclin promoter. In order to substantiate this conclusion, CAT activity was measured throughout differentiation of the two transformants. These measurements were consistent with the Northern blot data. While CAT activity was increased ~20-fold in the CAT-tub C transformants, it was only marginally affected in the CAT-tub V transformants (Figure 6). Similar results were obtained when the C and V constructs were targeted to the procyclin gene loci, except that in that case primary transcription was stimulated ~10-fold (data not shown). Interestingly, in both cases (insertion of the construct in either the tubulin or the procyclin locus) CAT activity of the C transformants was mainly stimulated between 12 and 18 h after triggering of differentiation (Figure 6; data not shown). Thus the change in gene expression occurred after the first cell division, later than the reprogramming of transcription (Pays *et al.*, 1993).

In conclusion, the results presented above all indicate that the stage-specific up- and down-regulation of CAT activity exerted by the 3′-end region of VSG mRNA can be accounted for by a modulation of mRNA abundance. Moreover, these controls can counteract those occurring at the transcriptional level. Similar conclusions may apply to the 3′-end region of procyclin mRNA, since the presence of this sequence downstream of the CAT gene in CAT-tub transformants (CAT-tub P) led to a clear increase in CAT mRNA amount in procyclic forms (Figure 5, lanes 13 and 14). It is not known if this region has a negative effect on CAT mRNA levels in the bloodstream stage, since so far we have been unable to obtain the bloodstream forms of the CAT-tub P transformants.

Stage-specific control of RNA stability

The nature of the mechanism by which the 3′-end region of VSG mRNA influences the level of CAT mRNA was approached by measuring the relative stability of this mRNA in both forms of the C and V transformants. This was performed by inhibiting RNA synthesis with actinomycin D, followed by measurement of the kinetics of CAT mRNA disappearance. Preliminary experiments performed by *in vivo* RNA labelling with [^{3}H]uridine indicated that 10 μg/ml actinomycin D was able to block >99% of transcription in both the bloodstream and procyclic forms. As shown in Figure 7, in bloodstream forms of the transformants CAT mRNA was at least three times more unstable without than with the 3′-end region (CAT mRNA half-life <5 min in C, ~17 min in V). In

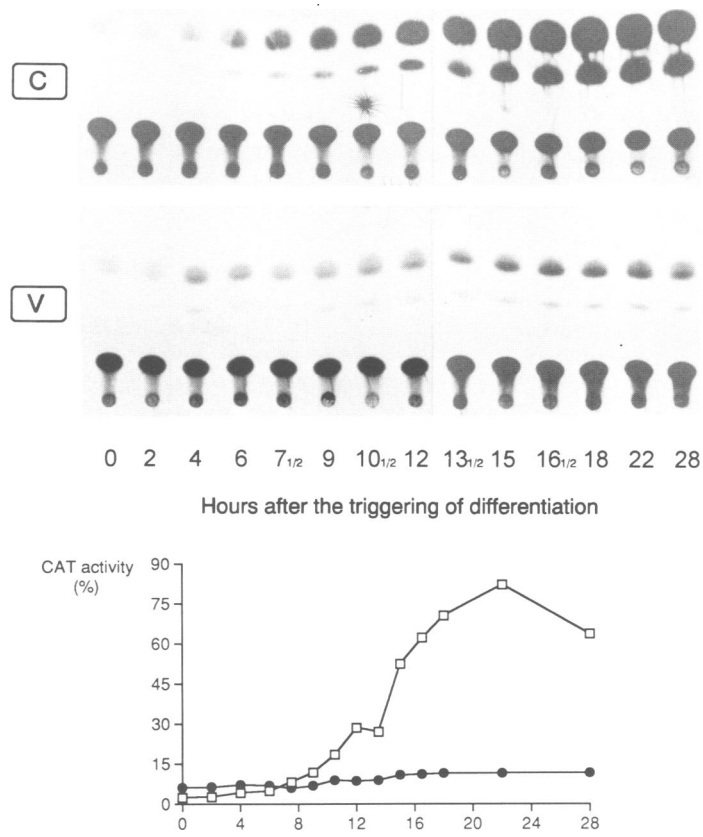

Fig. 6. CAT activity throughout *in vitro* differentiation of the CAT-tub C and V transformants. The percentage of chloramphenicol butyrylation is expressed as a function of time (h) elapsed after triggering of differentiation for C (squares) and V (dots).

this experiment the half-lives of actin and HYG^R mRNAs were found to be similar in the two transformants (~10 and 25 min respectively; see Figure 7). Thus it was clear that in the bloodstream forms the 3′-end region of VSG mRNA significantly increased stability of the mRNA. In contrast, in procyclic forms no significant difference in CAT mRNA stability was found between C and V (Figure 8). In this case the CAT mRNA half-life was ~30 min in both cases, slightly lower than that of HYG^R mRNA (35 min). These results were further supported by an analysis of a mutant form of the CAT-tub V transformant (CAT-tub VΔ14). In this transformant, for which we have the procyclic form only, the 3′-end region downstream of the CAT gene lacks the 14mer necessary for stage-specific regulation. Accordingly, in this case the amount of CAT mRNA was higher than in the CAT-tub V transformant (Figure 8, compare VΔ14 and V). Once again, this difference in mRNA amount did not appear to be linked to a difference in mRNA stability (Figure 8). Since primary transcription occurs at a similar rate in all transformants (see Figure 4), those differences in mRNA levels which are not linked to differential RNA stability can only be explained by differences in the efficiency of RNA maturation. Therefore, in procyclic forms the 3′-end region of VSG mRNA may alter the rate of RNA processing.

The difference in CAT mRNA stability observed in the bloodstream forms was also supported by UV irradiation experiments. Moderate irradiation of trypanosomes by UV leads to a transient inhibition of RNA degradation (Coquelet *et al.*, 1991). Under UV treatment which does not completely block transcription (typically 60 J/m^2) this inhibition leads to the preferential accumulation of RNAs that are normally the most unstable (Coquelet *et al.*, 1989, 1991). As shown in Figure 5 (lanes 15–20), in the bloodstream forms treated under these conditions CAT mRNA was found to increase more in CAT-tub C than in CAT-tub V (4.6-fold in C versus 1.4-fold in V after 1 min irradiation with 1 J/s/m^2). In contrast, HYG^R mRNA levels were found to be slightly and similarly increased in both cases (1.6- and 1.2-fold stimulation after 1 min UV for C and V respectively; Figure 5, lanes 15–20). Thus these results confirmed that in the bloodstream forms the 3′-end of VSG mRNA stabilizes CAT mRNA.

Discussion

A region from the 3′-end of VSG mRNA conferred both a 3-fold stimulation of reporter gene expression in the bloodstream form and the same extent of inhibition in the procyclic form, thus achieving a 10-fold difference between the two developmental stages of the parasite. Opposite results were obtained with the 3′-end of procyclin mRNA. The necessary and sufficient VSG mRNA sequence achieving these effects was defined as the 97 nt region located immediately upstream of the polyadenylation site. This sequence was actually found to be present at the 3′-end of the reporter mRNA. It includes both a polypyrimidine tract and conserved oligonucleotides and comprises the end of the VSG open reading frame. Given the length of this sequence, it is likely that complex

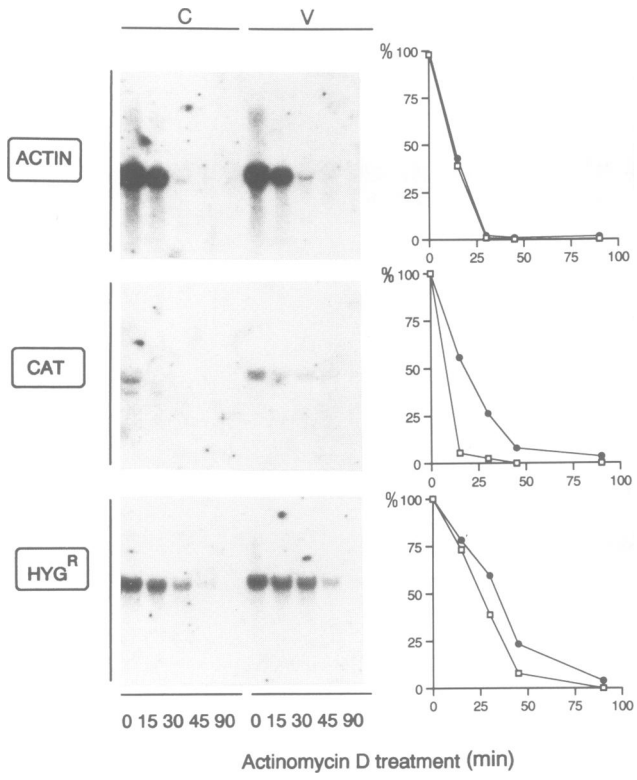

Fig. 7. Kinetics of RNA decay in bloodstream forms of the CAT-tub C and V transformants. Trypanosomes (2×10^8 cells) were inoculated into 100 ml Baltz medium supplemented with 10 μg/ml actinomycin D. They were harvested at the indicated times and total RNA was extracted using the trizol reagent (BRL). Aliquots (10 μg) of each RNA were subjected to Northern blot analysis using the indicated probes. Quantitative measurements were carried out by liquid scintillation counting and phosphorimager analysis and are plotted on the diagrams (black dots, C; white squares, V).

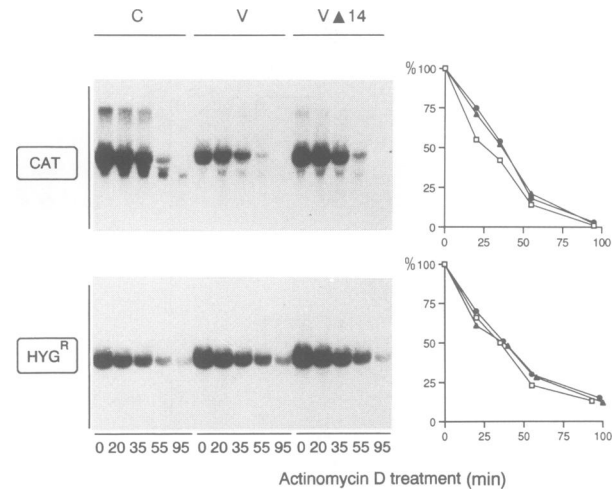

Fig. 8. Kinetics of RNA decay in the procyclic form of the CAT-tub C, V and VΔ14 transformants. Legend as in Figure 7. Black dots, C; white squares, V; black triangles, VΔ14.

protein and/or nucleic acid interactions are involved in the regulatory processes. The changes in gene expression seemed to occur entirely through modulation of steady-state mRNA levels, since the presence of the 3′-region induced changes in CAT mRNA levels which were consistent with the differences in CAT activity. The differences in mRNA levels were found to be independent of the rate of primary transcription and must thus be ascribed to post-transcriptional controls. In bloodstream forms they were linked to a significant difference in RNA stability, in contrast to procyclic forms, where no such difference was observed. Taken together, these results suggest that the 3′-region prevents mRNA degradation in the bloodstream forms, while in the procyclic form it inhibits maturation of the primary transcripts into mRNAs. In the letter case the site of polyadenylation was unaffected by deletions which achieved a loss of regulation, suggesting that inhibition of maturation is not linked to recognition of an alternative polyadenylation site.

The stage-dependent differences conferred on reporter mRNA levels by the 3′-end region of VSG and procyclin mRNAs are of the same order as those frequently observed for mRNAs of 'housekeeping' genes (examples in Gibson *et al.*, 1988; Revelard *et al.*, 1993). On this basis, the post-transcriptional modulation of gene expression described here could be taken as a model to explain the developmental regulation of gene expression in trypanosomes. However, it cannot account for the very important stage-dependent differences existing in mRNA levels for the major antigens. VSG mRNA seems to be completely absent from the procyclic form, while it represents as much as 5% of the polyadenylated transcripts of the bloodstream forms and a 60-fold difference was measured in procyclin mRNA levels between the procyclic and the bloodstream forms (E.Pays, unpublished data). Therefore, additional mechanisms must control the amounts of these mRNAs. At least one of these mechanisms acts at the level of RNA elongation. Although transcription is initiated in both the VSG and the procyclin units at both developmental stages, opposite temperature- and extracellular medium-dependent controls appear to modulate RNA elongation (Pays *et al.*, 1990; Rudenko *et al.*, 1994; Vanhamme *et al.*, 1995). Because it is the most distant from its promoter, the VSG gene is the most sensitive to this control. Arrest of transcription of the VSG gene is one of the earliest events triggered by a change in environmental conditions (Ehlers *et al.*, 1987; Kooter *et al.*, 1987; Pays *et al.*, 1990, 1993). Thus it is likely that the VSG gene is no longer transcribed in the procyclic form. Given these facts, the significance of the negative effect conferred by the 3′-end region of VSG mRNA in the procyclic form can be questioned. It may tentatively be explained by the fact that transcriptional controls operating on the VSG unit are not very tight. A complete repression of the VSG unit is only achieved after at least one cycle of cell division, since a low but significant level of VSG mRNA can still be detected up to 13 h after triggering of differentiation (Pays *et al.*, 1993), during a period when degradation of VSG mRNA is selectively accelerated (Ehlers *et al.*, 1987). Therefore, it is possible that the negative effect of the 3′-end region contributes to down-regulation of VSG mRNA levels in the transition period when full differentiation into the procyclic form is not yet achieved. Of course, the increase in RNA stability observed in the bloodstream form is consistent with selective accumulation of VSG mRNA at this stage. However, this effect alone cannot account for the exceptional quantity of VSG mRNA and other controls, possibly

at the level of *trans*-splicing (Kapotas and Bellofatto, 1993), must also be involved.

The 3′-UTR of procyclin mRNA contains a conserved 16mer whose sequence and folding are required to achieve gene expression (Hehl *et al.*, 1994). Clearly, the regulation conferred by this element is different from that described here. Indeed, in contrast to the complete UTR, the 16mer alone did not appear to modulate the amount of mRNA. This element possibly controls RNA translation (Hehl *et al.*, 1994).

Our experiments have revealed that when targeted to the tubulin locus, the procyclin promoter (a 505 bp fragment upstream of the start site of transcription) is not equally active in the two forms of the parasite. In the bloodstream forms it is bypassed by the RNA polymerase driven by the tubulin promoter, whereas it is active in the procyclic form, in accordance with a previous report (Lee and Van der Ploeg, 1990). These observations contrast with the results obtained when the same promoter is present in an episomal plasmid: in this case the promoter appeared to be similarly active in both forms (D.Jefferies and E.Pays, unpublished data). It also contrasts with results obtained with the VSG promoter which, when inserted in the tubulin locus, was bypassed by the tubulin polymerase in both the procyclic and bloodstream forms (Jefferies *et al.*, 1993). Therefore, it appears that the activity of trypanosome promoters is highly dependent on chromosomal context. Preliminary data suggest that this observation is linked to a stage-regulated transcription termination capacity of the promoter (M.Berberof and E.Pays, unpublished data).

Our results confirm that post-transcriptional controls, acting at the levels of mRNA maturation and stability, are very important in regulation of gene expression in trypanosomes. This observation is obviously linked to the particular organization of the genome in these organisms. As probably the majority of genes are clustered in polycistronic transcription units, any control at the levels of transcription initiation and/or elongation would imply that the different genes whose expression must be controlled by the same stimuli should be grouped in the same unit. This seems to be only true for units of the two major surface antigens, VSG and procyclin. However, even in these cases, the results reported here indicate that post-transcriptional regulation is also important.

Material and methods

Trypanosomes

Procyclic forms were obtained by *in vitro* cultivation of isolates from the midgut of flies infected with the EATRO 1125 stock of *T.brucei*. They were grown in SDM-79 medium (Brun and Schoenenberger, 1979) supplemented with 15% heat inactivated fetal calf serum. Cyclical transmission of the procyclic transformants through *Glossina morsitans morsitans* was performed as described previously (Jefferies *et al.*, 1993). Bloodstream forms were passaged in mice or cultivated according to Baltz *et al.* (1985). Procyclic forms were obtained from stumpy forms of the transformants by *in vitro* differentiation, as previously described (Pays *et al.*, 1993).

Plasmid constructs

The 138 bp *Hae*III–*Hin*dII fragment from the IsTat 1.1 gene in plasmid pTb1G.82 (Aline and Stuart, 1989) was ligated into the *Sma*I site of M13 mp19. Site-directed mutagenesis was performed with an Amersham kit following the manufacturer's instructions, then the 3′-end fragment was excised from M13 by *Eco*RI + *Hin*dIII digestion, blunt-ended and subcloned into the *Hpa*I site present downstream of the CAT gene in pD5 (Jefferies *et al.*, 1991) for transient CAT activity assays. For stable CAT expression, pD5 fragments containing the CAT gene and 3′-flanking region (*Ecl*136II–*Hpa*I and *Ecl*136II–*Bam*HI for C and V respectively) were ligated into pTSA-HYG2 (Sommer *et al.*, 1992) digested with either *Sac*I + *Bam*HI or *Xho*I, followed by end-blunting. The nucleotide sequences of these DNA fragments were checked twice, both after mutagenesis and after sub-cloning. The procyclin 3′-end region is a 480 bp *Nde*I–*Eco*RI fragment from plasmid pAP2 (Pays *et al.*, 1990). The tubulin 3′-end region is a 300 bp *Mlu*I–*Bgl*II fragment from plasmid pPT (Jefferies *et al.*, 1993). These fragments were blunt-ended for integration into the *Hpa*I site downstream of the CAT gene in pD5.

Electroporation

The procyclic forms were harvested at mid-log phase and washed once in Zimmerman post-fusion medium (Bellofatto and Cross, 1989). Cells (10^7) were resuspended in 500 µl of the same solution and mixed with 3 µg plasmid linearized by either *Mlu*I or *Nsi*I cleavage, to generate the CAT-tub or CAT-proc constructs respectively. Electroporation was performed by two pulses of 1.5 kV at 25 µF using a BioRad gene pulser. Electroporated trypanosomes were inoculated into 4 ml culture medium. Forty eight hours after electroporation, hygromycin was added to the culture medium, first at 25 and then at 100 µg/ml 5 days later. Transgenic trypanosomes could be detected 2–3 weeks after electroporation.

CAT assays

Electroporation was conducted as described above, except that the DNA amount was 30 µg. The measurement of CAT activity was performed as described previously (Jefferies *et al.*, 1991). All measurements were performed within a linear range of activity. The CAT activity of the control plasmid was typically 5×10^{-1} U/h in the procyclic form and 100-fold less in the bloodstream form.

DNA and RNA analysis

The procedures for DNA and RNA isolation, Southern and Northern blot hybridization and DNA cloning were as described (Pays *et al.*, 1990).

Run-on transcription

Run-on transcription assays were conducted as described by Murphy *et al.* (1987). The standard assay (1 ml) contained 500 µg DNA in nuclei, 12.5% (v/v) glycerol, 0.8 mg heparin, 5 mM spermidine, 5 mM $MgCl_2$, 2.5 mM dithiothreitol, 10 mM Tris–HCl, pH 8, 0.5 mM ATP, UTP, CTP and 1 mCi [α-^{32}P]GTP (2000 Ci/mmol). The nuclei were usually incubated for 30 min at 30°C.

UV irradiation

Bloodstream forms isolated by DEAE chromatography were incubated for 30 min in Baltz medium at 37°C at a concentration of $1–4\times10^7$ cells/ml. Samples (125 ml) were then irradiated at 254 nm (1 J/s/m^2) in sterile square dishes (22×22 cm) (Bio-Assay; Nunc, Roskilde, Denmark) for either 1 or 3 min with agitation. The irradiated cells were transferred to culture flasks and kept for 30 min at 37°C in the dark until centrifugation at 4°C. RNA was extracted from the pellets.

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